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A Bowman-Birk trypsin inhibitor with antiproliferative activity from Hokkaido large black soybeans

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Abstract: A trypsin inhibitor, with an *N*-terminal sequence highly homologous to those of 8-kDa Bowman-Birk trypsin inhibitors, was isolated from the seeds of Hokkaido large black soybeans. The trypsin inhibitor was unadsorbed on SP-Sepharose but adsorbed on DEAE-cellulose and Mono Q. It inhibited proliferation in breast cancer (MCF-7) cells and hepatoma (Hep G2) cells with an IC_{50} of 35 and 140 μM, respectively. The trypsin inhibitory activity of the inhibitor was completely preserved after exposure to temperatures up to 100 °C for 30 min and to the pH range 2–13 for the same duration. The trypsin inhibitor inhibited HIV-1 reverse transcriptase with an IC_{50} of 38 μM, but was devoid of antifungal activity toward *Fusarium oxysporum* and *Mycosphaerella arachidicola*.

Keywords: black soybean; trypsin inhibitor; antiproliferative

INTRODUCTION

The seeds of leguminous plants produce a variety of biologically active proteins including lectins [1], antifungal proteins [2,3], α-amylase inhibitors [4], arcelins [5], ribosome inactivating proteins [6], and protease inhibitors [7–15]. Most of these proteins play a role of defense, for instance, protection against pathogenic microorganisms and predatory insects [16–20]. In addition, they may exhibit potentially exploitable activities including antiproliferative, antitumor [21–26], and immunomodulatory [27] activities.

There are various classes of protease inhibitors. Trypsin inhibitors represent an important class of protease inhibitors, and can be divided into Kunitz-type [28–31]. Bowman-Birk type [7,11,15,32] and squash type [33] which have a molecular mass of approximately 20, 8, and 3 kDa, respectively. We report herein the isolation and characterization of a Bowman-Birk trypsin inhibitor from Japanese large black soybeans. It is an interesting protein because it is composed of two 8-kDa submits whereas most Bowman-Birk type inhibitors are monomeric. It also manifests potentially exploitable activities such as antiproliferative activity toward tumor cell lines. It has remarkable stability when subjected to a wide range of pH values and temperatures.

MATERIALS AND METHODS

Large black soybeans (Onishi beans Kuromame, *Glycine max*) from Hokkaido, Japan were soaked in distilled water, decoated, and then blended in distilled water. The resulting slurry was then centrifuged. To the supernatant obtained NH_4OAc buffer (pH 4.5) was added until the concentration reached 20 mM. The supernatant was loaded on a 2.5 × 20 cm column of SP-Sepharose (GE Healthcare). The unbound fraction was collected and chromatographed on a 2.5 × 20 cm column of DEAE-cellulose (Sigma) after dialysis against 20 mM Tris-HCl buffer (pH 7.8). After removal of unbound proteins, the column was eluted stepwise with 200 mM NaCl in the starting buffer to yield an active fraction which was subsequently further purified on Mono Q. After elution of unbound proteins, the column was washed with two consecutive linear NaCl concentration gradients (0–200 mM and then 200–1000 mM) in the starting buffer. The fraction eluted with nearly 200 mM NaCl was taken for gel filtration on Superdex 75 using an AKTA Purifier (GE Healthcare). The single absorbance peak from Superdex 75 represented purified trypsin inhibitor.

Electrophoresis, Molecular Mass Determination, and *N*-Terminal Sequence Analysis

The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [34]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of the isolated protein was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass of the protein. The *N*-terminal sequence of the protein was determined by using a Hewlett-Packard (HP) G1000A Edman degradation unit and an HP 1000 HPLC System.

Trypsin Inhibitory Assay

In the assay [35], 30 μl of test sample was added to 30 μl of trypsin (1 mg/ml) in assay buffer (50 mM Tris-HCl with 20 mM

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$CaCl_2$, pH 8.0). To make sure the inhibitors had reached equilibrium on binding to the proteases, the reaction mixture was incubated for 15 min at room temperature. After the incubation, 50 µl of the incubation mixture was transferred to 2 ml of the substrate solution N^{α}-benzoyl-L-arginine ethyl ester (0.08 mg/ml) (Sigma) in assay buffer. The inhibitory activity was then calculated as the difference between trypsin activity in the presence and the absence (control) of the inhibitor.

Calculation:

$$\text{trypsin inhibitory activity (U)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]/[\text{Abs}_{\text{control}} \times \text{Trypsin (mg)}]$$

Where, $\text{Abs}_{\text{control}}$ is the absorbance change in the absence of sample (control);
$\text{Abs}_{\text{sample}}$ is the absorbance change in the presence of sample; and
Trypsin is the amount of trypsin in the assay mixture.

One unit of trypsin inhibitor activity is defined as the activity capable of inhibiting 1 mg trypsin.

Assay of Antiproliferative Activity on Tumor Cell Lines

Breast cancer MCF-7 cells and hepatoma HepG2 cells were adjusted to 2×10^4 cells/ml. A 100 µl aliquot of this cell suspension was seeded to a well of a 96-well plate followed by incubation for 24 h. Different concentrations of the trypsin inhibitor were then added. After 72 h, 20 µl of a 5-mg/ml solution of [3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] (MTT) was spiked into each well. After 4 h, the plates were centrifuged at 324 g for 5 min. The supernatant was removed and 150 µl of dimethyl sulfoxide was added to dissolve the MTT-formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured [3].

Assay for HIV-1 Reverse Transcriptase Inhibitory Activity

The assay for HIV reverse transcriptase inhibitory activity was carried out according to instructions supplied with the ELISA kit from Boehringer Mannhein (Germany). The absorbance of the sample at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of reverse transcriptase activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the trypsin inhibitor was calculated as percent inhibition as compared to a control without the protein [3].

Assay of Antifungal Activity

The assay of the isolated trypsin inhibitor for antifungal activity toward *Botrytis cinerea, Mycosphaerella arachidicola* and *Fusarium oxysporum*, which are plant pathogens, was carried out using plates containing potato dextrose agar. The plates were incubated at 25°C until mycelial growth had enveloped disks containing the control and formed inhibition crescents around disks containing samples with antifungal activity [2,3].

RESULTS

Trypsin inhibitory activity resided in the fraction unbound on SP-Sepharose and subsequently bound to and eluted from DEAE-cellulose with 200 mM NaCl. This fraction was resolved on Mono Q into a number of absorbance peaks (Figure 1). The peak immediately preceding the highest peak was the only peak with trypsin inhibitory activity. It appeared as a single peak upon gel filtration on Superdex 75 (Figure 2). Its molecular weight was 16 kDa as judged by gel filtration (Figures 2 and 3) and 8 kDa by SDS-PAGE (Figure 4). Thus it was a dimeric protein. A 60-fold purification was achieved (Table 1). Its *N*-terminal sequence resembled Bowman-Birk trypsin inhibitors (Table 2). It does-dependently inhibited proliferation in MCF-7 cells (Figure 5) and Hep G2 cells (Figure 6) with an IC_{50} of 35 and 140 µM, respectively. HIV-1 reverse transcriptase was inhibited dose-dependently with an IC_{50} of 38 µM (Figure 7). It did not have antifungal

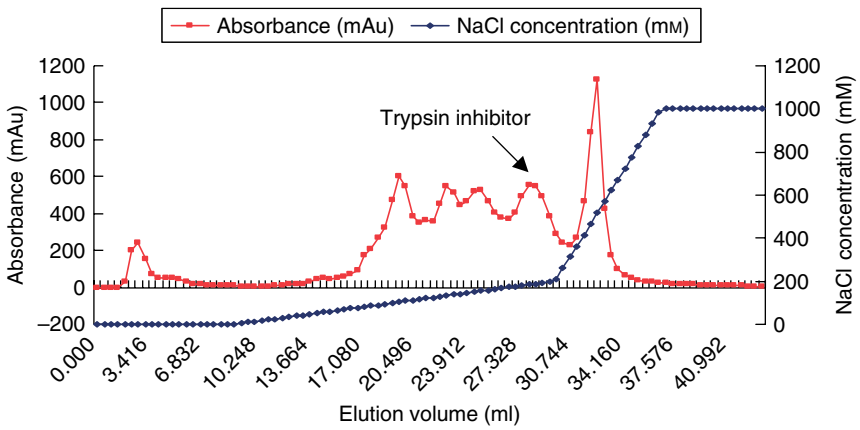

Figure 1 Profile of elution from Mono Q. Sample loaded: soybean extract previously unbound on SP-Sepharose and then bound on DEAE-cellulose.

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Table 1 Yields and activities after various steps of purification of trypsin inhibitor from Hokkaido large black soybeans

	Specific activity (U/mg)	Total protein (mg)	Total activity (U × 10^4)	Recovery of activity (%)	Purification (fold)
Crude extract	402	13 846	557	100	1
After SP-Sepharose	846	4534	384	69	2.1
After DEAE-Cellulose	2549	1098	280	50	6.3
After Mono Q	10 549	137	145	26	26.6
After Superdex 75	24 308	30	73	13	60.8

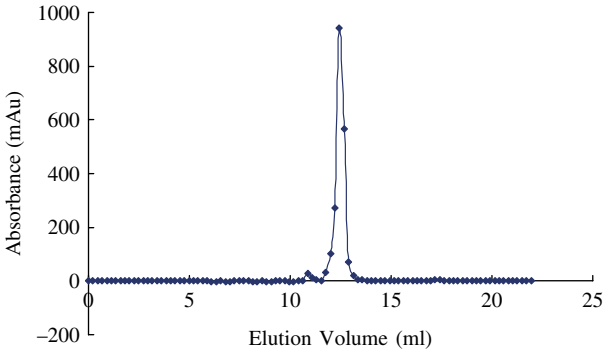

Figure 2 Profile of elution from Superdex 75. Sample loaded: active fraction from Mono Q column.

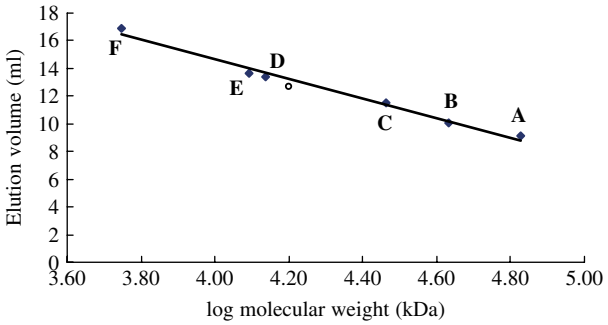

Figure 3 Molecular weight calibration curve for Superdex 75 column. The data points correspond to molecular markers from GE healthcare. A: Bovine serum albumin (67 kDa); B: Ovalbumin (43 kDa); C: Carbonic anhydrase (29 kDa); D: Ribonuclease A (13.7 kDa); E: Cytochrome c (12.4 kDa); F: Insulin: 5.6 kDa. The hollow circle represents the isolated trypsin inhibitor. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

activity toward *F. oxysporum* and *M. arachidicola* (data not shown).

DISCUSSION

Three chromatographic steps are required to yield a homogeneous preparation of trypsin inhibitor in this study. The steps include ion exchange chromatography on SP-Sepharose and DEAE-cellulose and fast protein liquid chromatography on Mono Q. The trypsin inhibitor preparation obtained after these three steps appeared to be homogeneous as evidenced by a single peak in gel filtration and a single band in SDS-PAGE.

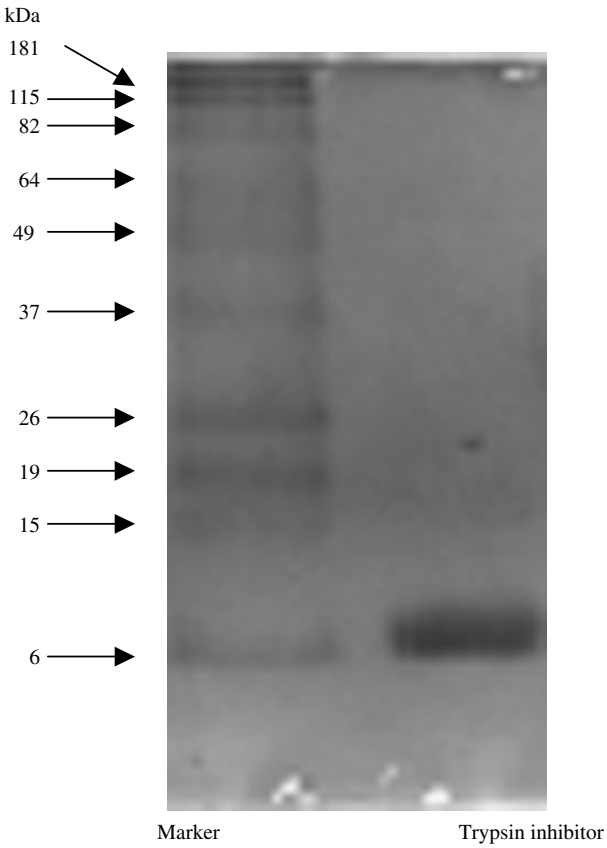

Figure 4 SDS-PAGE results. The subunit size indicated by the gel photo was around 8 kDa. The trypsin inhibitor is a 16-kDa protein with a subunit size 8 kDa.

Bowman-Birk trypsin inhibitors have a molecular mass of approximately 8 kDa. The trypsin inhibitor isolated from Japanese black soybeans in this study is a dimeric Bowman-Birk inhibitor since it has two

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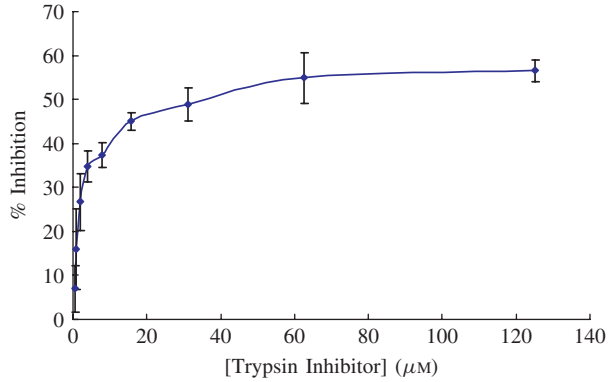

Figure 5 Antiproliferative effect of trypsin inhibitor from Hokkaido large black soybeans on MCF-7 cells. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

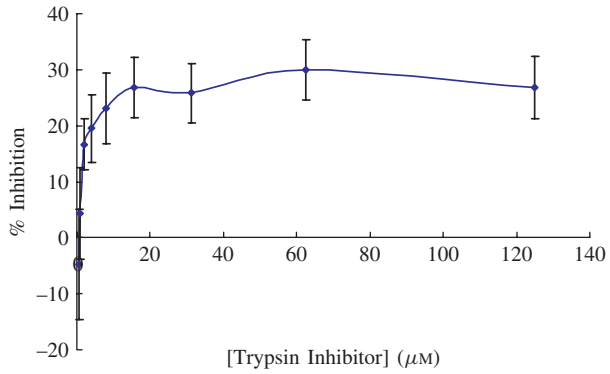

Figure 6 Antiproliferative effect of trypsin inhibitor from Hokkaido large black soybeans on HepG2 cells. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

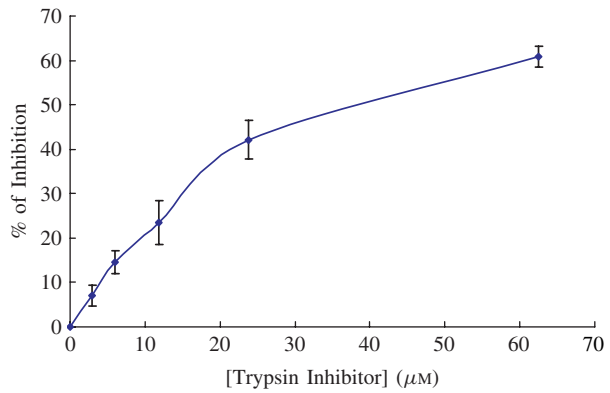

Figure 7 HIV-1 reverse transcriptase inhibitory activity of trypsin inhibitor from Hokkaido large black soybeans. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

8 kDa subunits. In this aspect it is similar to trypsin inhibitor from horse gram [36]. It has pronounced thermostability and pH stability and its activity remains untarnished after exposure to 100 °C and to a wide range of pH values. A highly stable Kunitz-type trypsin inhibitor has recently been isolated from the papaya [37].

Table 2 *N*-terminal sequence comparison of various trypsin inhibitors

	N-terminal sequence	% Identity
Hokkaido large black soybean trypsin inhibitor	1 DDESSKPCCDQCACT 15	–
Glycine max trypsin inhibitor	1 DDESSKPCCDQCACT 15	**100**
Glycine soja trypsin inhibitor	47 DDDSSKPCCDQCACT 61	**93**
Phaseolus augusti trypsin inhibitor	47 DEPSDSSKPCCDQCACT 63	**82**

Identical corresponding residues are underlined.

Phytoestrogens in soybeans such as genistein may have a protective effect against breast cancer [38]. Thus it is noteworthy that soybean trypsin inhibitor demonstrates antiproliferative activity toward MCF-7 breast cancer cells and that this activity is more potent than its antiproliferative activity against hepatoma cells. The antitumor and antiproliferative actions of leguminous trypsin inhibitors are well documented [21–26]. Some trypsin inhibitors manifest antifungal activity, e.g. those from the broad bean [7], lima bean [39], and the yellow soybean [39]. However, trypsin inhibitor from Japanese large black soybean is devoid of antifungal activity. The HIV-1 reverse transcriptase inhibitory potency of this trypsin inhibitor is within the range of potencies reported for anti-HIV natural products [40] and is higher than that reported for Kunitz-type trypsin inhibitor from soybean [41].

In summary, a trypsin inhibitor with some distinctive features including dimeric nature, pH, and thermal stability, has been isolated from Japanese large black soybeans.

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